ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

VIROLOGY

Virology 315 (2003) 292–302

www.elsevier.com/locate/yviro

Mutations in the C3 region of human and simian immunodeficiency virus envelope have differential effects on viral infectivity, replication, and CD4-dependency

Claas Otto,[a] Bridget A. Puffer,[b] Stefan Pöhlmann,[b] Robert W. Doms,[b] and Frank Kirchhoff[a,*]

[a] *Department of Virology, University of Ulm, Albert-Einstein-Allee 11, 89081 Ulm, Germany*

[b] *Department of Microbiology, University of Pennsylvania, 225 Johnson Pavilion, 3610 Hamilton Walk, Philadelphia, PA 19014, USA*

Received 24 April 2003; returned to author for revision 8 June 2003; accepted 24 June 2003

Abstract

Residues within the highly conserved C3 region of human and simian immunodeficiency virus (HIV, SIV) envelope proteins (Envs) bind directly to the cellular CD4 receptor. However, substitutions of D385, which is critical for CD4 engagement along with other changes such as G382R, G383R, frequently arise in SIV mac-infected macaques. We investigated the influence of substitutions in the SIVmac and HIV-1 C3 regions on viral entry, dependence on CD4, and replication. Mutations flanking the C3 region such as G382R or V388A enhanced and changes within the C3 region (i.e., G383R or D385N) impaired SIVmac infectivity. Several naturally occurring sequence variations in the SIVmac Env C3 region facilitated CD4-independent membrane fusion but abrogated viral replication, suggesting that efficient infection requires additional changes elsewhere in Env. Substitutions of S365R and D368G in the HIV-1 Env, which correspond to G382 and D385 in SIVmac Env, consistently impaired viral infectivity. In contrast, mutation of D368N resulted in a virus that could not spread in cells expressing low levels of CD4, but which replicated efficiently when high levels of CD4 were expressed. Thus, changes in the C3 region of HIV-1 or SIVmac Env can have differential effects on viral infectivity and CD4-dependency. We conclude that substitutions flanking the C3 region in SIVmac Env such as G382R or V388A represent one step toward adaptation to growth in target cells expressing low CD4 levels, whereas changes within the C3 region that disrupt CD4 binding might indicate the emergence of CD4-independent variants at later stages of infection, which could potentially broaden viral tropism.

Keywords: HIV-1; SIVmac; Envelope; C3 region; CD4-dependency

Introduction

Human immunodeficiency virus type 1 (HIV-1), the main causative agent of AIDS, and the related simian immunodeficiency viruses (SIV) infect target cells by binding of the external envelope glycoprotein (gp120) to the cellular CD4 receptor and subsequent interactions with chemokine receptors (Berger et al., 1999; Doms and Peiper, 1997). Mutational analysis has shown that binding of gp120 to CD4 involves a specific interaction between the second complementarity-determining region (CDR2) of CD4 and several conserved discontinuous epitopes in the HIV-1 envelope glycoprotein (Cordonnier et al., 1989; Kowalski et al., 1987; Moebius et al., 1992). Structural analysis of the gp120–CD4 complex demonstrated that residues D368 and E370 in the C3 region ($G_{366}GDPE_{370}$) of the HIV-1 Env make multiple contacts with amino acid residues F43 and R59 located in CDR2 of the CD4 receptor molecule (Kwong et al., 1998). The C3 region in gp120 is highly conserved between HIV-1 and SIV. Consequently, substitutions in the C3 region similar to D368N in the HIV-1 gp120 and the analogous D385N change in the SIVmac Env impair CD4 binding and viral infectivity (Morrison et al., 1995; Olshevsky et al., 1990; Ryzhova et al., 2002).

* Corresponding author. Fax: +49-731-50023337.
E-mail address: frank.kirchhoff@medizin.uni-ulm.de (F. Kirchhoff).

0042-6822/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S0042-6822(03)00513-0

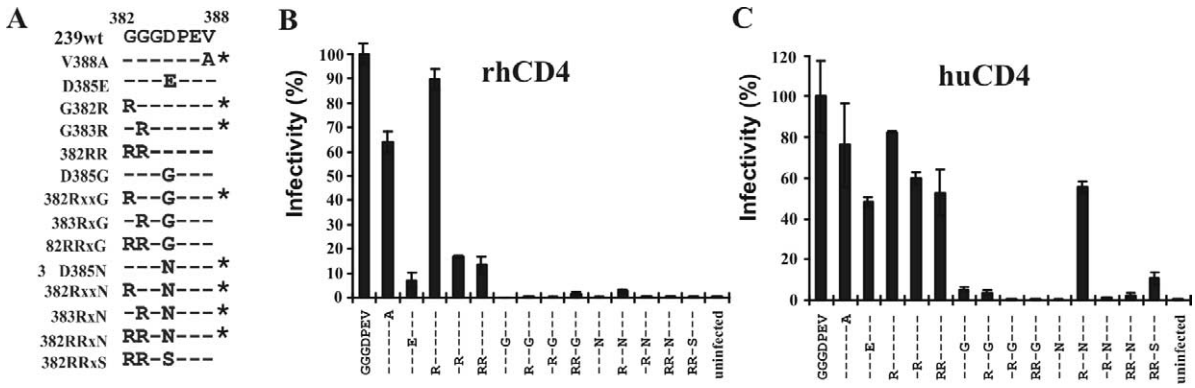

Fig. 1. (A) Amino acid substitutions in the SIVmac C3 region. Mutations detected in vivo in infected macaques are marked with an asterisk. Dashes indicate identity to the C3 sequence of SIVmac239 (Regier et al., 1990). The numbers above the alignment specify the position in the SIVmac239 Env. (B, C) Entry of SIVmac239 C3 variants into transiently transfected 293T cells coexpressing (B) rhesus or (C) human CD4 and human CCR5. 293T cells were transfected with plasmids expressing human CD4 and the entry cofactors indicated. At 1 day posttransfection the cells were detached from the plates, seeded in 48-well dishes, and infected in triplicate with replication-competent SIV carrying the luciferase gene in place of the *nef* gene. Virus stocks containing normalized amounts of p27 antigen (300 ng) were used for infection. Luciferase activities in the cellular extracts were measured at 3 days postinfection. Results were confirmed by two independent experiments.

In light of the critical role of these residues in CD4 binding, it is striking that substitutions of D385 in conjunction with other changes in the C3 region are frequently observed in tissues of rhesus macaques with simian AIDS and might be associated with a tropism for specific organs and altered pathogenicity (Anderson et al., 1993; Campbell and Hirsch, 1994; Kodama et al., 1993; Rudensey et al., 1995; Zhu et al., 1995). Notably, Envs from neurotropic, highly pathogenic SIVmac variants harboring the G383R and D385N changes in the C3 region, together with other substitutions in the C1 domain, the V1/V2 loop, and gp41, showed diminished CD4 binding and were able to use CCR5 in the absence of CD4 for cell–cell fusion (Ryzhova et al., 2002). Therefore, these changes might reflect the evolution of CD4-independent viral variants with a broadened cellular tropism. However, in this previous study it was not addressed whether the G383R and D385N substitutions alone are sufficient to mediate CD4-independent fusion and SIV infection. In contrast to the situation observed with SIV, changes in the HIV-1 C3 region are usually not selected for in HIV-1-infected individuals, suggesting that these alterations might compromise viral spread during all stages of infection.

In this article, we show that changes in the C3 region might facilitate CD4-independent fusion and that the G382R substitution in the SIVmac Env can partly compensate for the inactivating D385N mutation. In comparison, the corresponding S365R change in the HIV-1 NL4-3 Env did not restore viral infectivity. Substitution of D368N alone in the HIV-1 gp120 had differential effects on viral entry and replication. The D368N variant was inactive in cells expressing low levels of CD4 but replicated more efficiently than the parental NL4-3 clone in cells expressing high levels of CD4. These results suggest that specific changes in the HIV-1 C3 region might be advantageous for viral replication in primary cells or tissues that contain high surface levels of the CD4 receptor molecule. The alterations in or near the SIVmac C3 region selected in infected macaques seem to reflect a stepwise adaptation to CD4-independent entry.

Results

Substitution of G382R partially compensates for the D385N mutation in the SIVmac gp120 C3 region

Alterations in or near the C3 region became predominant in brain, colon, and other tissues derived from macaques displaying severe encephalomyelitis or enterocolitis after infection with the pathogenic SIVmac239 clone (Anderson et al., 1993; Campbell and Hirsch, 1994; Kodama et al., 1993; Rudensey et al., 1995; Zhu et al., 1995), which requires CD4 and CCR5 for efficient infection (Chen et al., 1997; Hill et al., 1997; Kirchhoff et al., 1997; Marcon et al., 1997). Since this region is important for CD4 binding, we generated a collection of 14 full-length, replication-competent SIVmac239 variants containing changes in the critical gp120 D385 residue and alterations in or near the C3 region that have been previously identified in vivo in infected macaques (Fig. 1A). Our goal was to assess the impact of these changes on viral infectivity, replication, and CD4-dependency. We used CCR5 and CD4 of human (hu) and rhesus macaque (rh) origin because, while both support SIVmac239 infection, subtle differences between these receptors might affect the efficiency with which some SIV strains infect cells, particularly in the absence of CD4 (Edinger et al., 1997). Sequence analysis confirmed that all variants differed from the parental SIVmac239 clone exclusively by the specific changes at amino acid residues 382, 383, 385, and 388 in the gp120. Western blot analysis

revealed that all mutant Env proteins were efficiently expressed and processed (data not shown).

The effect of alterations in the CD4-binding site on viral infectivity was first assessed by infection of sMAGI indicator cells (Chackerian et al., 1995) expressing human CD4 together with rhesus macaque coreceptors. We found that mutation of G382R and V388A flanking the critical D385 residue enhanced viral infectivity, while SIVmac239 containing either the G383R or the G382R mutation entered the cells with an efficiency comparable to 239wt (data not shown). Entry of all remaining mutants, including D385N, was at least five-fold reduced. Next, we infected 293T cells expressing rh or hu CD4 with the SIVmac C3 variants containing the luciferase reporter gene in place of *nef*. Only the G382R and V388A variants infected 293T cells expressing rhCD4 and huCCR5 with an efficiency comparable to wild-type SIVmac239 (239wt) (Fig. 1B). Substitutions of G383R, G382R/G383R, and D385E reduced SIVmac239 infectivity about 6- to 14-fold. All other C3 variants were grossly defective in viral entry into cells expressing rhCD4. Comparable results were obtained with cells coexpressing rhCD4 and huGPR15 (data not shown). Notably, SIVmac containing mutations of G383R or D385E entered efficiently into cells expressing huCD4 (Fig. 1C), whereas infection via rhCD4 was clearly diminished (Fig. 1B). This finding is unexpected because it has been suggested that the G383R change impairs SIVmac gp120 binding to huCD4 (Ryzhova et al., 2002).

The disruptive effect of the D385N substitution was partly compensated for by the G382R change. The D385N mutant virus showed $<0.5\%$ infectivity, relative to 239wt infection, irrespective of whether CD4 and coreceptor were of human or rhesus origin (Figs. 1B and C). In comparison, the 382RxxN variant infected CCR5 expressing cells via rhCD4 with $2.6 \pm 0.8\%$ efficiency and target cells expressing huCD4 with $55.6 \pm 2.9\%$ of 239wt efficiency (Fig. 1). Indeed, results from five independent experiments performed with coreceptors of both rhesus and human origin in various combinations revealed that the additional mutation of G382R enhanced the infectivity of the D385N variant for target cells expressing rhCD4 about 10- to 50-fold and for huCD4 between 500- and 3000-fold. In contrast, the adjacent mutation of G383R had no restorative effects on SIVmac D385N infectivity.

We next used an ELISA assay to investigate whether the infectivity of the G382R, G383R, D385N, and the RxxN C3 Env variants correlated with their ability to bind to rhCD4. The G382R change slightly reduced SIVmac gp120 binding to rhCD4 (Fig. 2). In contrast, the G383R change strongly impaired and mutation of D385N fully disrupted rhCD4 binding, thus correlating with their effects on viral infectivity. The introduction of the G382R substitution into the D385N Env background slightly increased CD4 binding (Fig. 2). Similar results were obtained when huCD4 was utilized in the binding assay (data not shown).

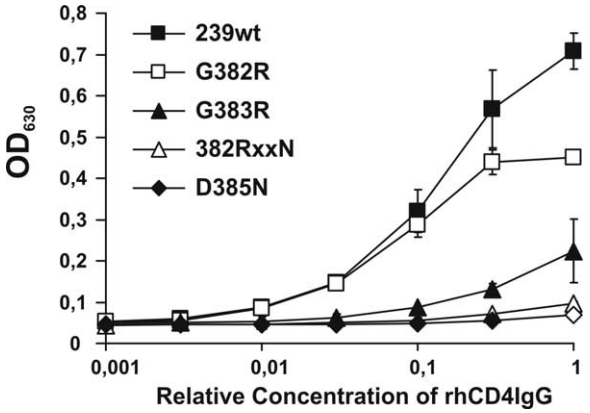

Fig. 2. Binding of SIVmac C3 variant Env proteins to rhCD4. Soluble gp120 proteins were generated and tested for rhCD4IgG binding in an ELISA as described under Materials and methods. OD_{630}, optical density at 630 nm. Similar results were obtained with huCD4.

Substitution of D385 induces CD4-independent cell–cell fusion but does not allow efficient infection of CD4-negative target cells

Ryzhova et al., (2002) have recently reported that SIVmac Envs containing changes of G383R or D385N show a CD4-independent phenotype. However, compared with the parental SIVmac239 clone, these envelopes contained additional changes in the C1 domain and in the V1/V2 loop as well as in gp41, which could contribute to their ability to utilize CCR5 in the absence of CD4. To address this, we investigated whether alterations in the SIVmac C3 Env region alone might allow CD4-independent fusion. As shown in Fig. 3, efficient fusion mediated by the 239wt, G383R, G382R, and V388A Env proteins required the presence of CD4. In contrast, analysis of the D385G, 382RR, 382RRxN, 382RxxN, 383RxN, D385N, and D385E mutant Envs revealed comparable levels of CCR5-mediated fusion in the presence or absence of CD4 (Fig. 3). The combination of the G383R and D385N mutations did not augment CD4-independence compared to the single D385N mutation. Notably, mutation of G382R and V388A generally enhanced cell–cell fusion, whereas all remaining C3 mutant Envs were less active than 239wt Env in the presence of CD4 and CCR5. The results show that alterations of D385 impair CD4-dependent and enhance CD4-independent cell–cell fusion, whereas substitutions of G382R and V388A flanking the C3 region generally enhance fusogenic activity.

The cell–cell fusion assay usually results in overexpression of both Env and coreceptor likely maximizing the assay sensitivity (Puffer et al., 2002; Rucker et al., 1997). We infected 293T cells transiently expressing rhesus or human CCR5 with luciferase reporter viruses to determine whether CD4-independent fusion was also observed under less artificial conditions. Infection of cells expressing both CD4 and CCR5 with 239wt virus typically resulted in luciferase activities $>100{,}000$ counts per second (cps) in the cellular

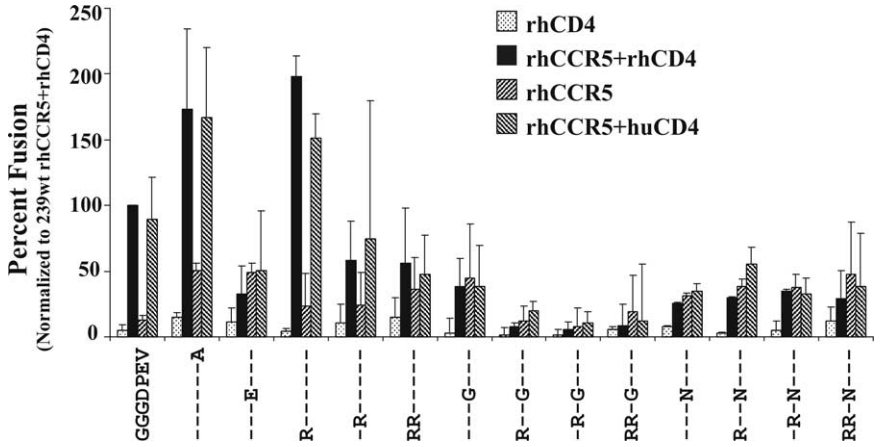

Fig. 3. CD4-independent CCR5 use by SIVmac C3 variant Envs. The fusion assay was performed by using quail QT6 target cells transiently expressing rhesus CD4 and CCR5 as described previously (Puffer et al., 2002; Rucker et al., 1997). Data from three experiments were averaged and the standard error of the mean was calculated. All values were normalized to levels of fusion detected for the 239wt Env in the presence of both CD4 and CCR5.

extracts. In contrast, the detectable levels of luciferase activity were generally <300 cps in the absence of CD4 even when very high viral multiplicities were used for infection with SIVmac 239wt or the C3 variants (data not shown). Taken together, our results demonstrate that changes in the SIVmac Env C3 region can facilitate CD4-independent cell–cell fusion but do not allow significant levels of viral infection or replication in the absence of CD4.

While mutations in the C3 region did not confer a detectable CD4-independent phenotype to SIVmac239, it is possible that they might lead to more efficient utilization of CD4 when this receptor is expressed at low levels. Indeed, more efficient use of limiting levels of CD4 has been linked to macrophage tropism in the SIV system (Banner et al., 2000; Mori et al., 2000). Therefore, we examined the ability of the mutant C3 SIVmac Envs to mediate fusion at limiting levels of rhCD4. As shown in Fig. 4A, comparable levels of cell–cell fusion were observed in cells transfected with 1.0 or 0.1 μg rhCCR5 expression plasmid, suggesting that co-receptor expression levels were not limiting. In contrast, fusion mediated by the 239wt, G382R, and V388A Env proteins declined as the level of CD4 decreased. In comparison, the G383R, 383RxN, and D385N Envs were less sensitive toward the decrease in CD4 levels (Fig. 4A). In agreement with the findings mentioned above, the 383RxN and D385N Envs were less and the G382R and V388A Env proteins were more active in mediating cell–cell fusion. Next, we tested the sensitivity of G382R Env to neutraliza-

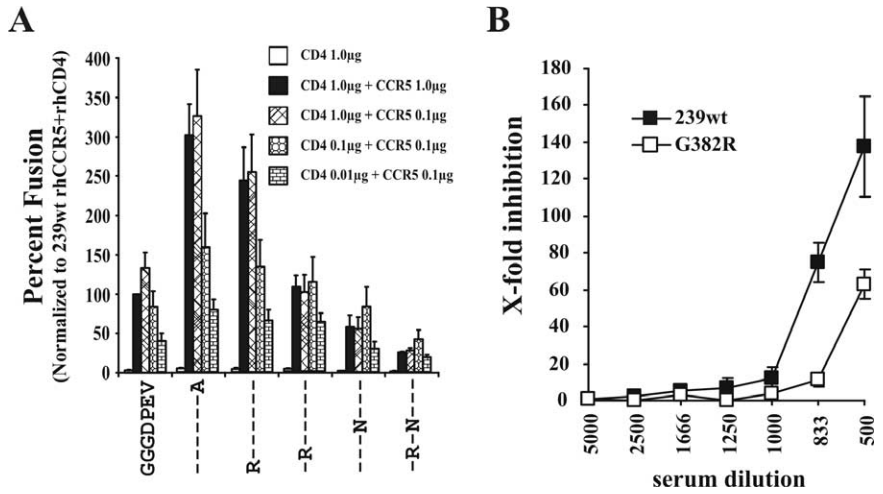

Fig. 4. Analysis of fusion at limiting levels of rhCD4 and neutralization sensitivity. (A) Target cells were transfected with the designated amounts of plasmids expressing rhCD4 and rhCCR5 and fusions assays were performed in duplicate as described in the legend to Fig. 4. (B) The SIVmac239 G382R variant is less susceptible to neutralization by MAb SK3 than 239wt. The ability of MAb SK3 to inhibit infection of P4-CCR5 cells by SIVmac 239wt or G382R was tested at the indicated dilutions of antibody. Infections were performed in triplicate; the standard deviation is shown. Similar results were obtained in two independent experiments.

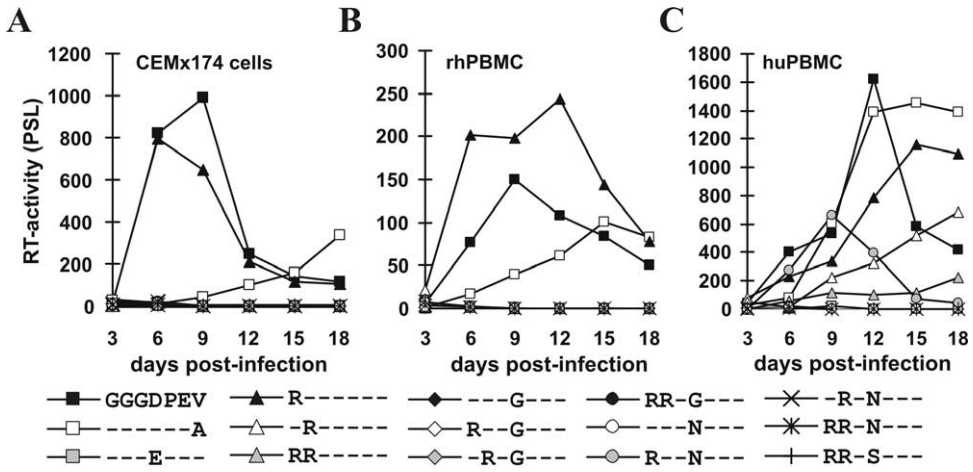

Fig. 5. Replication of SIVmac239 C3 variants in (A) CEMx174 cells and in (B) rhesus or (C) human PBMC. Cells were infected with 5 ng p27 containing virus derived from transfected 293T cells. Virus production was monitored by assays of reverse transcriptase (RT) activity in the culture supernatants at the indicated days post-infection. Similar results were obtained in three independent experiments. PSL, photo-stimulated light emission.

tion by a monoclonal antibody to CD4 in P4-CCR5 indicator cells expressing both CCR5 and CD4. We found that the G382R mutant virus was less sensitive than 239wt to neutralization with this mAb (Fig. 4B). These results indicate that the G382R and V388A substitutions flanking the SIVmac Env C3 region modulate CD4 utilization and decrease susceptibility to inhibition by mABs to CD4.

Finally, we analyzed the replicative capacity of the SIVmac239 C3 mutants in several cell types. Only the 239wt virus and the G382R variant replicated with 239wt-like efficiency in the T-B hybrid cell line CEMx174 expressing CD4 and GPR15 (Fig. 5A). Despite high activity in infectivity and fusion assays, the V388A variant replicated less efficiently and with delayed kinetics. All remaining C3 mutants were grossly defective in viral replication. This result is consistent with our finding that these SIVmac239 C3 variants enter inefficiently into 293T cells, coexpressing CD4 and GPR15 (data not shown). Likewise, only the G382R and V388A variants replicated to detectable levels in rhPBMC (Fig. 5B). However, the G383R, 382RR, and 382RxxN variants, which were consistently inactive in rhPBMC, replicated in huPBMC albeit with delayed kinetics and reduced efficiency compared to 239wt (example shown in Fig. 5C). This is in agreement with with our observation that these changes in the C3 region reduce SIVmac entry via huCD4 less severely than infection via rhCD4 (Figs. 1B and C). All single substitutions at position 385 (D385E, N, or G) completely disrupted viral spread in PBMC irrespective of their species origin. Thus, consistent with the infectivity-data, replication of the RxxN variant in huPBMC confirms that mutation of G382R can partially compensate for the disruptive D385N change. Most remarkably, however, the results clearly demonstrate that several changes in the C3 region selected in vivo render the virus completely inactive for replication in primary rhPBMC.

Substitution of D368N in the HIV-1 Env C3 region affects the threshold level of CD4 cell-surface expression required for viral replication

While the crystal structure of SIV gp120 has not been solved, the highly conserved nature of both CD4 and the C3 region in gp120 prompted us to examine HIV-1 gp120, for which a structure is available. Residue D368 in the HIV-1 gp120, which is analogous to residue D385 in SIVmac239, makes direct contact with amino acids F43 and R59 in the CD4 molecule (Kwong et al., 1998) (Fig. 6A). Analogous to the changes in the SIVmac C3 domain (Fig. 1A), we introduced mutations of S365R, G366R, and D368N or G in the Env glycoprotein of the X4-tropic HIV-1 NL4-3 clone (Fig. 6B). This molecular HIV-1 clone was utilized because it has been already been thoroughly characterized by different research groups. First, we infected several cell lines expressing different levels of CD4 with NL4-3 mutants containing the luciferase gene in place of *nef*. CD4 surface expression levels were estimated by the mean fluorescence intensities (m.f.) after parallel staining with the CD4-specific mAb OKT4. All changes in the C3 region disrupted HIV-1 entry into P4-CCR5 cells expressing low levels of CD4 (m.f. 14.2) in conjunction with CXCR4 (Fig. 6C). In contrast, the D368N substitution reduced entry into U373-MAGI-CXCR4CEM cells (m.f. 24.6) only to about 50% compared to NL4-3wt (Fig. 6D). Unlike the D385N mutation in the SIVmac239 Env (Figs. 1B and C), no disruptive effect of the corresponding D368N mutation in HIV-1 Env was observed in transiently transfected 293T cells expressing high levels of huCD4 (m.f. 282.9) and CXCR4 (Fig. 6E). Thus, the effect of the D368N change on HIV-1 infectivity was dependent on the CD4 expression levels of the target cells. In comparison, D368G generally disrupted HIV-1 infectivity. Unlike the corresponding change in the SIVmac CD4 binding site, the additional substitution of

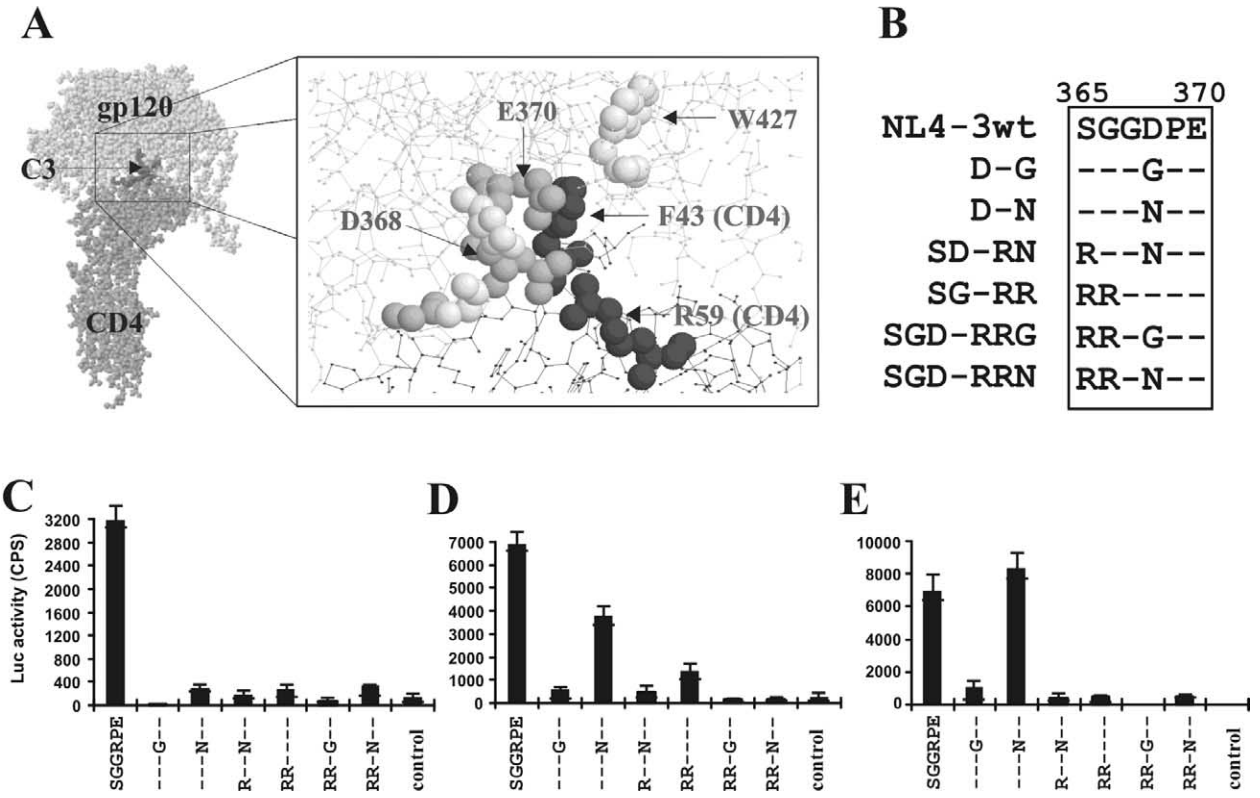

Fig. 6. HIV-1 NL4-3 C3 variants analyzed and their infectivity for target cells expressing different CD4 levels. (A) Localization of the gp120 C3 region in the Envelope CD4 complex (Kwong et al., 1998). Residues in Env mutated in this study and amino acids F43 and R59 in CD4 are indicated. (B) Mutations in the HIV-1 C3 region analyzed. Dashes indicate sequence identity and the numbers above the alignment refer to the amino acid position in the NL4-3 Env sequence. (C) P4-CCR5 cells, (D) U937-MAGI-CXCR4CEM indicator cells, and (E) transiently transfected 293T cells coexpressing human CD4 and X4 were infected in triplicate with replication-competent HIV carrying the luciferase gene in place of the *nef* gene. Virus stocks containing normalized amounts of p24 antigen (300 ng) were used for infection. Luciferase activities in the cellular extracts were measured at 3 days postinfection. Results were confirmed in two independent experiments.

S365R had no restorative effects. In contrast the SD-RN variant was clearly less infectious than the D368N mutant virus (Figs. 6D and E). These results suggest that the SIVmac and HIV-1 Env proteins might interact with CD4 in a somewhat different manner. None of the NL4-3 C3 variants replicated above background levels in CEMx174 cells (Fig. 7A). In contrast, the D368N and SG-RR mutants spread efficiently in PM1 cells albeit with delayed kinetics (Fig. 7B). In huPBMC only NL4-3 and D368N showed efficient levels of replication with the spread of the D368N variant being more dependent on the PBMC donor (examples shown in Fig. 7C). In agreement with a previous study examining a similar mutation of D368E (Platt et al., 1998) our results suggest that the D368N substitution in the HIV-1 C3 region impairs HIV-1 infectivity at low CD4 expression levels, but has less disruptive effects at high levels of CD4 expression.

To further test this hypothesis, we infected Jurkat-T cells expressing low or high levels of CD4 (Cortes et al., 2002) with NL4-3 wt and the different C3 mutants. FACS analysis confirmed that Jurkat-T high-CD4 cells expressed about 10-fold higher levels of surface CD4 than low-CD4 cells (m.f. 218 versus 24) but similar levels of CXCR4 (m.f. 156 versus 168). As shown in Fig. 8A, only the parental HIV-1 NL4-3 clone replicated to detectable levels when Jurkat-T low-CD4 cells were infected with virus stocks containing 5 ng p24 antigen. After infection with a 10-fold higher dose, some inefficient and delayed replication of the D368N variant was also observed (Fig. 8B). In contrast, the D368N mutant replicated consistently with accelerated kinetics and higher efficiency than NL4-3wt in Jurkat cells expressing high levels of CD4 (Figs. 8C and D). The D385G and SD-RN mutants replicated to some extent in Jurkat cells expressing high levels of CD4 after inoculation with virus stocks containing 50 ng p24 antigen (Fig. 8D). However, in agreement with the grossly defective viral entry (Fig. 6), spread of these variants was strongly delayed and inefficient. Thus, the D368N mutation in the HIV-1 C3 region is associated with impaired viral infectivity and replication at low levels of CD4 cell-surface expression but accelerates viral replication in cell expressing high levels of CD4.

Discussion

In this study, we investigated the effect of naturally occurring sequence alterations in the SIVmac Env C3 re-

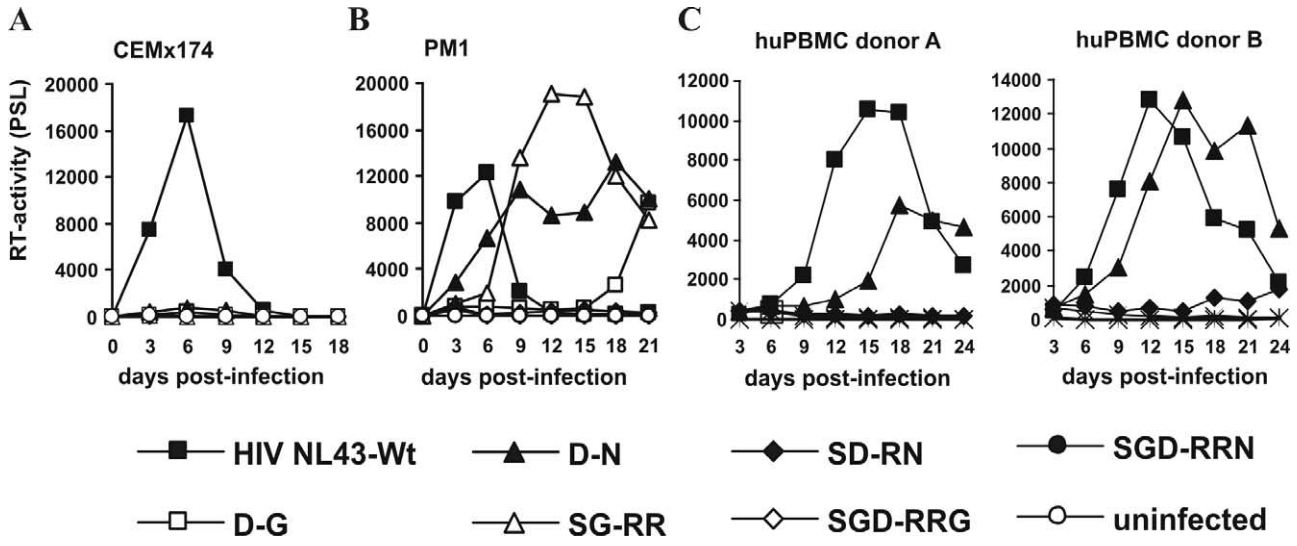

Fig. 7. Replication of HIV-1 NL4-3 C3 variants. (A) CEMx174 cells, (B) PM1 cells, and (C) huPBMC from two different donors were infected with 5 ng p24 containing virus derived from transiently transfected 293T cells. Virus production was monitored as described in the legend to Fig. 4. Comparable results were obtained in two independent experiments and when 10-fold higher viral doses were used for infection.

gion and corresponding changes in the HIV-1 Env on viral infectivity and replication. Mutations were introduced into the well-characterized SIVmac239 and HIV-1 NL4-3 molecular clones. SIVmac239 had previously been utilized in studies describing unusual sequence variations in the C3 region upon infection of rhesus macaques (Anderson et al.,

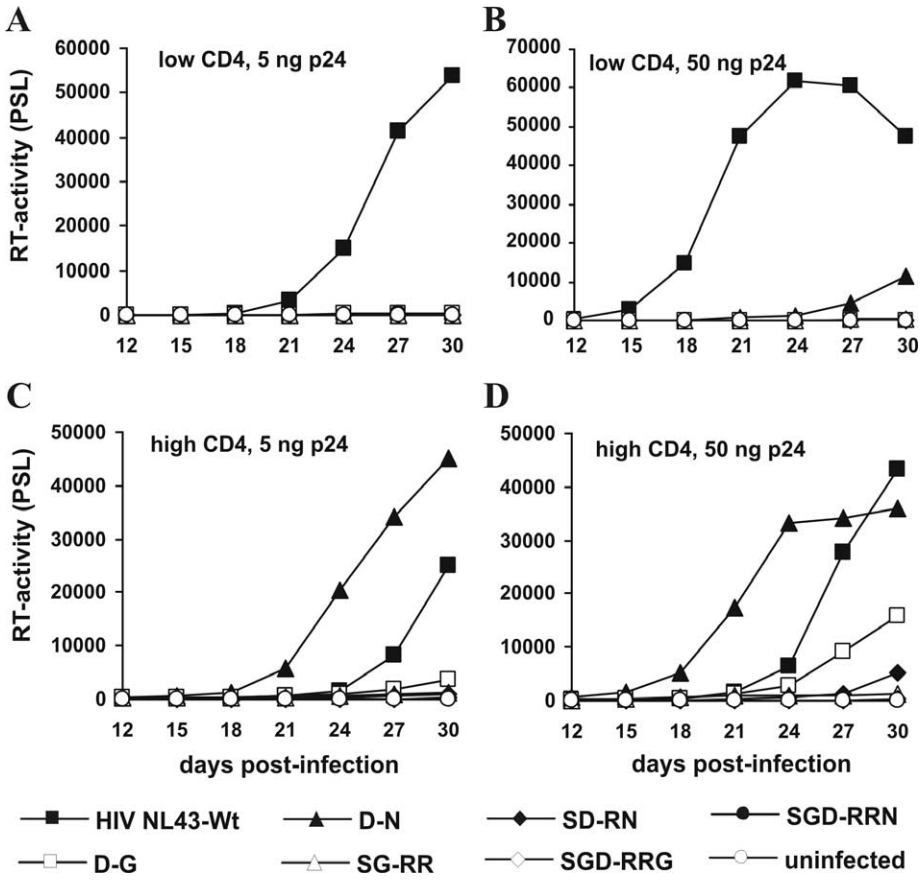

Fig. 8. Replication of HIV-1 NL4-3 C3 variants in Jurkat cells expressing differential levels of CD4. Jurkat cells expressing low (A, C) or high (B, D) levels of CD4 were infected with virus stocks containing 5 or 50 ng p24 antigen. Results were reproduced in two independent experiments with independent virus stocks and all constructs were verified by sequence analysis.

1993; Campbell and Hirsch, 1994; Kodama et al., 1993; Rudensey et al., 1995). These amino acid changes included the substitution of a central aspartic acid residue at position 385, which likely binds CD4 directly. We demonstrate that the D385N substitution in the SIVmac Env C3 region facilitates CD4-independent cell–cell fusion but abrogates viral infection and that this disruptive effect is partially compensated by the adjacent G382R substitution. The majority of the C3 substitutions selected in vivo severely impaired viral infectivity and replication, when introduced into SIVmac239. These findings together with a recent report by Ryzhova et al. (2002) suggest that additional mutations elsewhere in the SIVmac genome must be selected to allow effective CD4-independent viral spread in infected macaques.

The amino acid changes at positions 382, 383, 385, and 388 in SIVmac 239 Env selected in experimentally infected macaques had the following differential effects on viral replication: (i) Individual mutations of G382R or V388A frequently enhanced SIVmac infectivity in both the absence and the presence of CD4. This result is consistent with the observation that G382R is sufficient to strongly increase SIVmac239 replication in cells expressing low levels of CD4, similar to rhesus macaque alveolar macrophages (Bannert et al., 2000; Mori et al., 1992, 1993, 2000), and is present in some CD4-independent SIVmac isolates (Edinger et al., 1997; Flaherty et al., 1997; Zhu et al., 1995). (ii) The 382RR, G383R, D385E, and RxxN changes reduced SIVmac infection but rendered the virus not completely inactive. (iii) The remaining SIVmac239 C3 variants D385G, 382RxxG, 383RxG, 382RRxG, D385N, 383RxN, 382RRxN, and 382RRxS were grossly defective in both viral entry and replication. However, in agreement with a recent study (Ryzhova et al., 2002), most of these changes enhanced CD4-independent cell–cell fusion. Nevertheless, none of these SIVmac239 C3 variants entered efficiently into any of the cell types tested or replicated in primary rhesus-derived peripheral blood mononuclear cells (PBMC). These data indicate that results obtained with highly sensitive cell–cell fusion assays can have limited relevance for viral infectivity and demonstrate that changes elsewhere in Env are required to restore efficient viral replication.

What drives the selection of these changes in infected rhesus macaques? Further studies are required to obtain definitive proof, but we feel that most likely the alterations in the SIVmac Env C3 region reflect the stepwise adaptation of SIVmac toward effective CD4-independent entry. Single substitutions of G382R have frequently been observed in SIVmac isolates derived from brain, lymph node, or lung (Flaherty et al., 1997; Hill et al., 1997; Rudensey et al., 1995; Zhu et al., 1995) and this change is sufficient to increase viral replication in macrophages (Mori et al., 1992, 1993). This viral property might be due to efficient utilization of low densities of CD4 for efficient infection of target cells (Bannert et al., 2000; Mori et al., 2000). Our results suggest that G382R increases the general fusogenic activity of SIVmac Env and alters the interaction with CD4, resulting in decreased inhibition by an anti-CD4 mAB. In contrast, individual substitutions of D385N or D385G consistently disrupted SIVmac infectivity. Remarkably, however, these changes were frequently observed in conjunction with the G382R substitution in infected macaques (Kodama et al., 1993; Zhu et al., 1995). Thus, the sequence and functional data suggest that G382R might be initially selected to enhance infection at lower levels of CD4 expression. Subsequently, the D385N or D385G substitutions in conjunction with additional changes in the V1/V2 and V3 region or elsewhere in Env are selected to enhance CD4-independent coreceptor binding and thus promote the efficient infection of target cells that either lack or express low levels of CD4. In support of this assumption changes across the full length of Env have been detected in CD4-independent SIVmac variants and contribute to infection of macrophages which express low levels of CD4 (Anderson et al., 1993; Mori et al., 1992; Puffer et al., 2002). This hypothesis would explain why alterations in the SIVmac C3 region were mainly observed in tissues, expressing low amounts of CD4 such as brain or lung.

Several SIVmac C3 variants analyzed in this study utilized huCD4 more effiently for viral infection than rhCD4 (Fig. 2). This finding resembles our previous results showing that SIVmac efficiently utilizes human but not rhesus STRL-33 for viral entry (Pohlmann et al., 2000) and further emphasizes the importance of using receptors derived from the appropriate species when studying Env function. We investigated several CD4 mutants to elucidate why the SIVmac RxxN Env variant infected cells expressing huCD4 with about 30-fold higher efficiency than cells expressing rhCD4 (Fig. 2). Altogether, human- and rhesus-derived CD4 differ in 35 amino acid residues. For example, R59 in huCD4, which forms salt bridges with N368 in HIV-1 Env (Kwong et al., 1998) and most likely also with N385 in SIVmac Env, is replaced by a lysine residue in rhCD4. We found that the R59K substitution in huCD4 reduced SIVmac RxxN entry by 50%. However, the reciprocal change in rhCD4 did not significantly enhance infection by the mutant virus (C. Otto and F. Kirchhoff, unpublished observations). Thus, it remains to be clarified why huCD4 is used more efficiently by the RxxN Env than rhCD4.

In contrast to the findings in SIVmac-infected rhesus macaques, alterations in the HIV-1 C3 region in infected humans were never documented. Unlike the corresponding G382R change in SIVmac Env, substitution of S365R in HIV-1 Env both individually and in conjunction with D368N reduced viral infectivity and delayed or impaired viral replication. Consistent with our functional data, suggesting that S365R is not advantageous for viral spread, an arginine is not present at this position in any of the Env sequences in the HIV-1 sequence database. We found that substitutions of D368N or D368G both impaired HIV-1 infectivity. Our results are in agreement with previous studies demonstrating that D368 is important for CD4 binding

and makes direct contact with residues F43 and R59 of the receptor (Fig. 7A) (Kwong et al., 1998; Olshevsky et al., 1990). However, in contrast to the corresponding D385N change in the SIVmac C3 region, which always impaired viral infectivity, the disruptive effect of the D368N substitution was dependent on the levels of CD4 expression. Our data resemble those of Platt et al. (1998), who found that wild-type HIV-1 efficiently infects cells expressing a wide range of cell-surface CD4 levels, whereas a D368E mutant virus requires substantially higher levels of CD4 for efficient infection. In contrast, heterologous changes such as D368G or D368P completely disrupt HIV-1 infection. Extending the previous study, we found that the HIV-1 NL4-3 D368N variant can spread efficiently in human PBMC. Notably, this mutant virus replicated with accelerated kinetics and greater efficiency than wild-type HIV-1 NL4-3 in Jurkat cells expressing high surface levels of CD4. It has been shown that CD4 inhibits virus release and might prevent Env incorporation into virion or result in the incorporation of Env/CD4 complexes that are inactive in mediating HIV-1 infection (Bour et al., 1999; Ross et al., 1999). The lower affinity of the D368N Env for CD4 could reduce these inhibitory effects and might allow the virus to replicate efficiently in cells expressing high quantities of CD4. Accordingly, such variants might also have a selective advantage in primary cells or tissues expressing high CD4 levels.

In summary, we show that arginine substitutions flanking the critical aspartic acid residue in the Env C3 region enhance SIVmac infectivity but impair HIV-1 infectivity. This likely explains why these mutations are only observed in SIV mac infection. Changes of D385G or D368G generally disrupted SIVmac and HIV-1 infectivity, respectively. In comparison, an asparagine at position 385 consistently impaired SIVmac infection, whereas the corresponding change in HIV-1 Env had no disruptive effects at high levels of CD4 expression. The results suggest that HIV-1 and SIVmac Env interact with CD4 in a somewhat different manner. The disruptive D385N or D385G changes in SIVmac Env might only be selected as a last step in the adaptation to CD4-independent entry. We are currently investigating whether changes in the V1/V2 and V3 region observed in SIVmac Env C3 variants increase the efficiency of CD4-independent infection.

Materials and methods

Mutant construction

The SIVmac239D385E variant and all HIV-1 NL4-3 C3 mutants were generated by splice overlap extension PCR essentially as described (Pohlmann et al., 1999). The remaining SIVmac239 C3 mutants (Fig. 1A) were generated using mutagenic primers containing the *Mro*I restriction site at position 7761 of the proviral genome (Regier and Desrosiers, 1990). Briefly, the 239wt *env* region was amplified using p5outV3 (5′-GAGTCTTGTGACAAACA-3′) and p3-G382R (5′-AGGTAACTTCCGGATCTCCTCC**TCG**AGG-AGCC-3′); p3-G383R (5′-AGGTAACT-TCCGGATCTC-C**TCT**TCCAGGA-3′); p3-382RR (5′-AGGTAACT-TCCGGAT CTCC**TCT-TCT**AGGAGCC-3′); p3-D385G (5′-AAGGTAACTTCCGG**ACC**TCCTCCTC-3′); p3-382RxxG (5′ - AGGTAACTTCCGG**ACC**TCCTCC**TCT**AGGAGCC-GT-3′); p3-383RxG (5′-AGGTAACTTCCGG**ACC**TC-C**TCT**TCCAGGAGCCGT-3′); p3-382RRxG (5′-AG-GTAAC-TTCCGG**ACC**TCC**TCTTCT**AGGAGCCGT-3′); p3-D385N (5′-GAAGGTAACTTCCGG-**ATT**TCCTC-CTCCAGGAGCC-3′); p3′-382RxxN (5′-AGGTAACTTC-CGG**ATT**TCCTCC**TCT**AGGAGCCGT-3′); p3-383RxN (5′-AGGTAACTTCCGG**ATT**TCC**TCT**TCCAGGA-GC-3′); p3′-382RRxN (5′-AGGTAACTTCCGG**ATT**TCC**TCTT-CT**AGGAGCCGT-3′); or pF52 (5′-TTCCGGATTTCCT-CTTCTAGGAGC-3′); and p5V388A (5′-GAGGAGATC-CGGAA**GCT**A-CCTTCATG-3′). Changes compared to the 239wt sequence are in bold; the *Mro*I site is underlined. PCR products were digested with *Xho*I and *Mro*I or *Cla*I (V388A), gel purified, and cloned into a modified full-length 239wt provirus (Pohlmann et al., 1999). All PCR-derived inserts were sequenced to ensure that only the intended changes were present. Replication-competent luciferase reporter viruses were generated by standard cloning techniques.

Coreceptor expression vectors

Vectors expressing human and rhesus macaque CCR5 were provided by Nathaniel Landau and Preston Marx through the AIDS Research and Reference Program, Division of AIDS, NIAID, NIH. Expression vectors for human GPR15 were kindly provided by Dan Littman (Skirball Institute for Molecular Medicine, New York, NY). Vectors expressing rhesus macaque GPR15 were generated as described (Pohlmann et al., 1999, 2000).

Virus stocks

Generation of virus stocks was performed by the calcium phosphate method as described (Deng et al., 1996). Briefly, 293T cells were transfected with 10 μg of the full-length proviral SIVmac239 or HIV-1 NL4-3 clones. After overnight incubation, the medium was changed and virus was harvested 24 h later. Viral stocks were aliquoted and frozen at −80°C. p24 and p27 antigen concentrations, respectively, of viral stocks were quantitated with an SIV p27 antigen capture ELISA obtained through the National Institutes of Health AIDS Research and Reference Reagent Program.

Cells

CEMx174, PM1, and Jurkat cells were maintained in RPMI 1640 medium supplemented with 10% FCS. sMAGI cells (Chackerian et al., 1995), P4-CCR5, and 293T cells

were grown in DMEM medium supplemented with 10% FCS and antibiotics. Generation of Jurkat cells expressing different levels of CD4 has been described previously (Cortes et al., 2002). Peripheral blood mononuclear cells were isolated using lymphocyte separation medium (Organon Teknika Corp., Durham, NC) stimulated for 3 days with 2 μg phytohemagglutinin per milliliter and cultured in RPMI 1640 medium with 20% FCS and 100 U/ml IL-2. Supernatants were collected at 3- or 4-day intervals and virus production was measured by reverse transcriptase assay as described (Potts, 1990).

Entry, fusion, and antibody inhibition assays

To determine coreceptor activity of the SIV mac or HIV-1 C3 mutants, 293T cells were transiently cotransfected with CD4 and coreceptor-expression plasmids. After overnight incubation the medium was changed and the cells were seeded in 48-well dishes. The following day cells were infected with stocks of the luciferase-reporter viruses containing 300 ng of p27 antigen, respectively, in a total volume of 0.5 ml. At 3 days after infection cells were lysed and the luciferase activities in 20 μl cell lysates were determined using a commercially available kit (Promega). The fusion assay was performed as described elsewhere (Puffer et al., 2002, Rucker et al., 1997). Briefly, effector 293T cells were infected with a vTF1.1 recombinant vaccinia virus expressing the T7 RNA polymerase followed by $CaCl_2$-mediated transfection of an Env expression plasmid. QT6 target cells were cotransfected with plasmids expressing CCR5 and/or CD4 and a T7 luciferase plasmid expressing the luciferase gene under the control of the T7 promoter. One day after transfection, the effector cells were overlaid on the target cells and incubated at 37°C for 8 h to allow the formation of syncytia. To quantify cell–cell fusion, the cells were lysed, mixed with luciferase substrate (Promega), and counted in a luminometer (Wallac). For antibody inhibition experiments, P4-CCR5 cells were preincubated with the various dilutions of mAb SK-3 pure or CD4-V4 (Becton–Dickinson) for 1 h prior to infection with virus stocks containing 100 ng of p27 antigen. Infectivity was quantitated 3 days postinfection using the Galacto-Light Plus chemiluminescence reporter assay kit (Tropix, Bedford, MA) as recommended by the manufacturer.

FACS

Cells were detached by treatment with 5 mM EDTA and washed with FACS staining buffer (1% calf serum, 0.1% azide, in PBS). CD4 expression was determined by staining with the anti-CD4 mAb OKT4 conjugated with phytoerythrin (PE) (Becton–Dickinson). Washing was repeated, and the cells were resuspended in FACS buffer and staining was analyzed on a FACScanner (Becton–Dickinson) with CellQuest software.

ELISA

Supernatants were produced by infection of 293T cells followed by transfection with the designated Env expression plasmid. Forty-eight hours posttransfection, the medium was harvested and filtered to remove cell debris. The supernatant was applied to 96-well ELISA plates coated with 100 μl *Galanthus nivalis* lectin (Vector Laboratories), blocked with 2% blotto in TBS (10 μg/mL in capture buffer), and incubated overnight. Plates were then washed and incubated with serially diluted RhCD4IgG containing supernatant for 2 h at room temperature. Plates were again washed and incubated with anti-rabbit horseradish peroxidase (Promega) at 1:10,000 dilution for 1 h. Plates were then washed and developed with TM Blue substrate (KPL) and absorbance at OD_{630} was determined using a Dynex MLX plate reader with Revelation software.

Acknowledgments

A number of reagents were obtained through the AIDS Research and Reference Program, Division of AIDS, NIAID, NIH. We thank Nathaly Finze and Mandy Krumbiegel for expert technical assistance and Ingrid Bennett for critical reading. We also thank Bernhard Fleckenstein and Thomas Mertens for constant support and encouragement. This work was supported by Deutsche Forschungsgemeinschaft, SFB-466, and the Wilhelm-Sander Foundation. B.A.P. was supported by NIH Grant F32 AI 10577, and R.W.D. was supported by a Burroughs Wellcome Fund Translational Research Award, the Pediatric AIDS Foundation, and NIH Grants AI40880 and AI45378.

References

Anderson, M.G., Hauer, D., Sharma, D.P., Joag, S.V., Narayan, O., Zink, M.C., Clements, J.E., 1993. Analysis of envelope changes acquired by SIV mac239 during neuroadaption in rhesus macaques. Virology 195, 616–626.

Bannert, N., Schenten, D., Craig, S., Sodroski, J., 2000. The level of CD4 expression limits infection of primary rhesus monkey macrophages by a T-tropic simian immunodeficiency virus and macrophagetropic human immunodeficiency viruses. J. Virol. 74, 10984–10993.

Berger, E.A., Murphy, P.M., Farber, J.M., 1999. Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. Annu. Rev. Immunol. 17, 657–700.

Bour, S., Perrin, C., Strebel, K., 1999. Cell surface CD4 inhibits HIV-1 particle release by interfering with Vpu activity. J. Biol. Chem. 274, 33800–33806.

Campbell, B.J., Hirsch, V.M., 1994. Extensive envelope heterogeneity of simian immunodeficiency virus in tissues from infected macaques. J. Virol. 68, 3129–3137.

Chackerian, B., Haigwood, N.L., Overbaugh, J., 1995. Characterization of a CD4-expressing macaque cell line that can detect virus after a single replication cycle and can be infected by diverse simian immunodeficiency virus isolates. Virology 213, 386–394.

Chen, Z., Zhou, P., Ho, D.D., Landau, N.R., Marx, P.A., 1997. Genetically divergent strains of simian immunodeficiency virus use CCR5 as a coreceptor for entry. J. Virol. 71, 2705–2714.

Cordonnier, A., Montagnier, L., Emerman, M., 1989. Single amino-acid changes in HIV envelope affect viral tropism and receptor binding. Nature 340, 571–574.

Cortes, M.J., Wong-Staal, F., Lama, J., 2002. Cell surface CD4 interferes with the infectivity of HIV-1 particles released from T cells. J. Biol. Chem. 277, 1770–1779.

Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., Di Marzio, P., Marmon, S., Sutton, R.E., Hill, C.M., Davis, C.B., Peiper, S.C., Schall, T.J., Littman, D.R., Landau, N.R., 1996. Identification of a major co-receptor for primary isolates of HIV-1. Nature 381, 661–666.

Doms, R.W., Peiper, S.C., 1997. Unwelcomed guests with master keys: how HIV uses chemokine receptors for cellular entry. Virology 235, 179–190.

Edinger, A.L., Mankowski, J.L., Doranz, B.J., Margulies, B.J., Lee, B., Rucker, J., Sharron, M., Hoffman, T.L., Berson, J.F., Zink, M.C., Hirsch, V.M., Clements, J.E., Doms, R.W., 1997. CD4-independent, CCR5-dependent infection of brain capillary endothelial cells by a neurovirulent simian immunodeficiency virus strain. Proc. Natl. Acad. Sci. USA 94, 14742–14747.

Flaherty, M.T., Hauer, D.A., Mankowski, J.A., Zink, M.C., Clements, J.E., 1997. Molecular and biological characterization of a neurovirulent molecular clone of simian immunodeficiency virus. J. Virol. 71, 5790–5798.

Hill, C.M., Deng, H., Unutmaz, D., Kewalramani, V.N., Bastiani, L., Gorny, M.K., Zolla-Pazner, S., Littman, D.R., 1997. Envelope glycoproteins from human immunodeficiency virus types 1 and 2 and simian immunodeficiency virus can use human CCR5 as a coreceptor for viral entry and make direct CD4-dependent interactions with this chemokine receptor. J. Virol. 71, 6296–6304.

Kirchhoff, F., Pohlmann, S., Hamacher, M., Means, R.E., Kraus, T., Uberla, K., Di Marzio, P., 1997. Simian immunodeficiency virus variants with differential T-cell and macrophage tropism use CCR5 and an unidentified cofactor expressed in CEMx174 cells for efficient entry. J. Virol. 71, 6509–6516.

Kodama, T., Mori, K., Kawahara, T., Ringler, D.J., Desrosiers, R., 1993. Analysis of simian immunodeficiency virus sequence variation in tissues of rhesus macaques with simian AIDS. J. Virol. 67, 6522–6534.

Kowalski, M., Potz, J., Basiripour, L., Dorfman, T., Goh, W.C., Terwilliger, E., Dayton, A., Rosen, C., Haseltine, W., Sodroski, J., 1987. Functional regions of the envelope glycoprotein of human immunodeficiency virus type 1. Science 237, 1351–1355.

Kwong, P.D., Wyatt, R., Robinson, J., Sweet, R.W., Sodroski, J., Hendrickson, W.A., 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature 393, 648–659.

Marcon, L., Choe, H., Martin, K.A., Farzan, M., Ponath, P.D., Wu, L., Newman, W., Gerard, N., Gerard, C., Sodroski, J., 1997. Utilization of C-C chemokine receptor 5 by the envelope glycoproteins of a pathogenic simian immunodeficiency virus, SIVmac239. J. Virol. 71, 2522–2527.

Moebius, U., Clayton, L.K., Abraham, S., Harrison, S.C., Reinherz, E.L., 1992. The human immunodeficiency virus gp120 binding site on CD4: delineation by quantitative equilibrium and kinetic binding studies of mutants in conjunction with a high-resolution CD4 atomic structure. J. Exp. Med. 176, 507–517.

Mori, K., Ringler, D.J., Desrosiers, R.C., 1993. Restricted replication of simian immunodeficiency virus strain 239 in macrophages is determined by env but is not due to restricted entry. J. Virol. 67, 2807–2814.

Mori, K., Ringler, D.J., Kodama, T., Desrosiers, R.C., 1992. Complex determinants of macrophage tropism in env of simian immunodeficiency virus. J. Virol. 66, 2067–2075.

Mori, K., Rosenzweig, M., Desrosiers, R.C., 2000. Mechanisms for adaptation of simian immunodeficiency virus to replication in alveolar macrophages. J. Virol. 74, 10852–10859.

Morrison, H.G., Kirchhoff, F., Desrosiers, R.C., 1995. Effects of mutations in constant regions 3 and 4 of envelope of simian immunodeficiency virus. Virology 210, 448–455.

Olshevsky, U., Helseth, E., Furman, C., Li, J., Haseltine, W., Sodroski, J., 1990. Identification of individual human immunodeficiency virus type 1 gp120 amino acids important for CD4 receptor binding. J. Virol. 64, 5701–5707.

Platt, E.J., Wehrly, K., Kuhmann, S.E., Chesebro, B., Kabat, D., 1998. Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1. J. Virol. 72, 2855–2864.

Pohlmann, S., Lee, B., Meister, S., Krumbiegel, M., Leslie, G., Doms, R.W., Kirchhoff, F., 2000. Simian immunodeficiency virus utilizes human and sooty mangabey but not rhesus macaque STRL33 for efficient entry. J. Virol. 74, 5075–5082.

Pohlmann, S., Stolte, N., Munch, J., Ten Haaft, P., Heeney, J.L., Stahl-Hennig, C., Kirchhoff, F., 1999. Co-receptor usage of BOB/GPR15 in addition to CCR5 has no significant effect on replication of simian immunodeficiency virus in vivo. J. Infect. Dis. 180, 1494–1502.

Potts, B.J., 1990. "Mini" reserve transcriptase, in: Aldovini, A., Walker, B.D. (Eds.), Techniques in HIV Research. Stockton Press, New York, pp. 103–106.

Puffer, B.A., Pohlmann, S., Edinger, A.L., Carlin, D., Sanchez, M.D., Reitter, J., Watry, D.D., Fox, H.S., Desrosiers, R.C., Doms, R.W., 2002. CD4 independence of simian immunodeficiency virus Envs is associated with macrophage tropism, neutralization sensitivity, and attenuated pathogenicity. J. Virol. 76, 2595–2605.

Regier, D.A., Desrosiers, R.C., 1990. The complete nucleotide sequence of a pathogenic molecular clone of simian immunodeficiency virus. AIDS Res. Hum. Retroviruses 6, 1221–1231.

Ross, T.M., Oran, A.E., Cullen, B.R., 1999. Inhibition of HIV-1 progeny virion release by cell-surface CD4 is relieved by expression of the viral Nef protein. Curr. Biol. 17, 613–621.

Rucker, J., Doranz, B.J., Edinger, A.L., Long, D., Berson, J.F., Doms, R.W., 1997. Cell-cell fusion assay to study role of chemokine receptors in human immunodeficiency virus type 1 entry. Methods Enzymol. 288, 118–133.

Rudensey, L.M., Kimata, J.T., Benveniste, R.E., Overbaugh, J., 1995. Progression to AIDS in macaques is associated with changes in the replication, tropism, and cytopathic properties of the simian immunodeficiency virus variant population. Virology 207, 528–542.

Ryzhova, E., Whitbeck, J.C., Canziani, G., Westmoreland, S.V., Cohen, G.H., Eisenberg, R.J., Lackner, A., Gonzalez-Scarano, F., 2002. Rapid progression to simian AIDS can be accompanied by selection of CD4-independent gp120 variants with impaired ability to bind CD4. J. Virol. 76, 7903–7909.

Zhu, G.W., Liu, Z.Q., Joag, S.V., Pinson, D.M., Adany, I., Narayan, O., McClure, H.M., Stephens, E.B., 1995. Pathogenesis of lymphocytetropic and macrophage-tropic SIVmac infection in the brain. J. Neurovirol. 1, 78–91.